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(54) **A novel diagnostic marker for splicing variants of genes associated with neurological function**

(57) Methods are described for detecting the presence or absence of a four amino acid motif (VRXQ) in expressed proteins that arise from aberrant alternative splicing of premRNA in genes associated with normal neurological function which are useful for detecting neurodegenerative disease. The presence of these variants

suggest that mutational events in these genes have occurred. Methods to measure the levels of gene expression of such genes to detect neurodegenerative disease are provided. Nucleotide sequences and intron-exon junctional sequences of examples of this splicing variant and probes for detecting this variant which are useful as diagnostic reagents are also provided.

Description

BACKGROUND OF THE INVENTION

5 In eukaryotes, the initial transcription of genomic DNA into RNA proceeds in the nucleus and yields a contiguous full-length reverse complementary heteronuclear RNA (hnRNA) primary transcript. The hnRNA contains regions or contiguous blocks of nucleotide sequence that end up in the final mRNA (exons) interspersed between "intervening" nucleotide sequences (introns) that do not. In addition to adenylyl methylation and polyadenylation, these hnRNAs are extensively modified in a process referred to as RNA "splicing" wherein discontinuous exons are joined and the inter-

10 vening intron precisely deleted as an RNA "lariat" from the final mature mRNA transcript (B. Rushkin et al. Cell 1984, 38:317; R.A. Padgett et al. Science 1984, 225:898). RNA splicing is a complex process involving large protein-RNA assemblies called spliceosomes that coordinate the concerted excision and ligation events to yield intron-free mRNAs (M.M. Konarska and P.A. Sharp Cell 1987, 49:763; R. Reid et al. Cell 1988, 53:949; T.A. Steitz Sci. Am. 1988, 258:56). In normal RNA processing, the resultant mRNA reflects the linear sequence orientation of the exons in the hnRNA; however all exons do not end up in the final transcripts. Rather, several of the resultant mRNAs have only certain exons that result from "alternatively spliced" hnRNA, wherein discontinuous intron-exon junctions are spliced to bring for instance exon1 and exon 4 into juxtaposition rather than exon1 and exon 2. Therefore, several mRNAs may arise from one gene sequence or hnRNA. Not all possible combinations of exons are normally represented in actual mRNA pools arising from one hnRNA as determined by mRNA, cDNA and protein analyses. As an example with three exons (Figure 20 1), while seven combinations are possible (exon1-exon2-exon3, exon1-exon2, exon1-exon3, exon2-exon3, exon1, exon2, or exon3) perhaps only two (exon1-exon2-exon3 and exon1-exon3) may actually result and be expressed at any appreciable level. These alternatively spliced transcripts are sometimes referred to as "variants". However, for purposes of this invention splice "variant" refers to heretofore unrepresented or expressed mRNAs arising from potential alternative splice sites that result from genomic mutation altering the structure of the hnRNA so that these splices now occur.

25 The location of splice sites in an hnRNA primary transcript can be determined by comparing the sequences of the corresponding genomic DNA with that of cDNA prepared by copying the corresponding mature mRNA. Any discontinuities between the genomic DNA and cDNA sequences mark the exon-intron boundaries. Such analyses of a number of different RNAs have defined moderately -short "consensus" sequences at the intron-exon boundaries in pre-mRNA and a tendency for a pyrimidine-rich region just upstream of the 3' splice junction (Figure 2). The only universally conserved nucleotides are the first two (GU) and last two (AG) in the intron (Figure 2), though there is a propensity for AG at the 5' exon termini and an initial G at the 3' exon. Only 30-40 nucleotides in the center portion of introns are necessary for efficient splicing. There is also a conserved A within the context of the pyrimidine rich region of the intron (Figure 2) (.PyrPyrPurAPyrnAG; where Pyr is a pyrimidine and Pur is a purine nucleotide) which is the branch point where the cleaved 5' exon-intron junction loops back to form the "lariat" splicing intermediate (Padgett et al. Science 1984, 225:898). Genetic point mutations that delete or alter these conserved intronic nucleotides (5' GU, 3' AG, or branch point A) would eliminate these splice junctions and prevent normal splicing yielding aberrantly truncated transcripts or transcripts where this exon is deleted and another downstream exon spliced in, that normally may not be spliced in.

40 A final mechanism for splice variation occurs when several GU or AG dinucleotide motifs occur near consensus intron splice regions of 5' exon-intron or 3' intron-exon boundaries, respectively, such that the splicing system may sometimes not correctly distinguish the correct splice site resulting in alternate protein product some of which may be non-functional or aberrant.

Multiple examples of splice variations exist, many of which are associated with diseases or related disorders. Previous genetic linkage studies have shown a G to A mutation at the 3' splice junction of exon 8 of the gene encoding lysosomal acid lipase. Defects in this gene are associated with cholesterol ester storage disease that result in premature arteriosclerosis, hepatomegaly, and elevated LDL cholesterol (U. Seedorf et al. Arterioscler. Throb. Vasc. Biol. 1995, 15: 773-778). Two mutations at the exon 1/intron 1 boundary altered the hepatic specific splicing of the human hydroxymethylbilane synthase gene (third enzyme in heme biosynthetic pathway) and resulted in an enzyme with half-normal activity (K.H. Astrin Human Mutat. 1994, 4:243-252). Deficiency of this enzyme activity eventually results in acute intermittent porphyria (AIP), an autosomal dominant inborn error of metabolism in which life-threatening attacks are precipitated by ecogenetic factors. Molecular cloning of cDNA and genomic DNA have provided probes allowing presymptomatic detection of these gene defects. In Menke's disease, a point mutation at the - 2 exonic position of a splice donor site in the middle of the gene causes exon-skipping and activation of a cryptic splice acceptor site (S.G. Kaler et al. Nat. Genet. 1994, 8:195-202). Exon skipping of the entire exon 19 results from a G to A point mutation at the 5' donor site of intron 19 in muscle phosphofructokinase deficiency (T. Hamaguchi Biochem. Biophys. Res. Comm. 1994, 202:444-449). Aberrant RNA splicing from a splice site mutant in the interleukin-2 receptor gamma (gL2-R) gene results in the generation of an abundant non-functional gL2-R containing a small intronic insertion and a second

mutant form with 5-fold lower affinity (J.P. DiSanto et al. Proc. Natl. Acad. Sci. 1994, 91:9466-9470). These isoforms produce an atypical form of an X chromosome-linked severe combined immunodeficiency disease.

The presence of splice variants can be used as diagnostic markers of diseases associated with genetic mutations. For example, the expression of the exon 6 splice variant (v6) of the cell adhesion molecule CD44 is correlated with the expression of the tumor suppressor gene p53. Both have been shown to be markers of tumor progression in colorectal cancer (J.W. Mulder et al. Gut 1995, 36:76-80; Y. Matsumura Lancet 1992, 340:1053-1058). Asymptomatic carriers of the acute intermittent porphyria were identified by identification of a mutant allele containing a CG to CT transversion at the exon1/intron 1 boundary via in vitro amplification of DNA followed by hybridization of the target sequence to allele-specific oligonucleotides.

Accordingly, splicing variants have been observed in several gene loci and several diseases. Identification of these variants has proven to be especially useful in diagnosis and detection of asymptomatic carriers.

SUMMARY OF THE INVENTION

A novel insertional motif that arises from splice mutations or alternative utilization of cryptic or less preferred splice donor sites has now been identified. These splicing variations result in the in-frame insertion within a normal protein sequence of four amino acids, valine-arginine-X-glutamine (VRXQ); where X is a hydrophilic amino acid. This motif has been identified in splice variants of a receptor, an enzyme, and a putative channel protein, all of which are involved in normal neurological functioning. Identification of this motif allows for screening of genes and gene products for splice variations.

A method for the detection of this motif in expressed proteins in vitro or in situ with the use of specific antisera, polyclonal or monoclonal antibodies is provided. A method for the detection of allele-specific genetic mutations using selected oligonucleotides with standard hybridization-based detection techniques is also provided. A method for diagnosing Alzheimer's Disease (AD) by detecting differences in levels of transcripts having the VRXQ insertion or proteins encoded therefrom is further provided. A preferred embodiment of such method for detecting AD provides for the detection of Familial Adult Onset Alzheimer's Disease (FAD).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic of potential alternative splicing with 3 exons and 4 introns.

Figure 2 is a schematic of the consensus exon-intron-exon structure and sequence.

Figure 3 provides the sequence of the VRSQ variant of the presenilin 1 gene. SEQ ID NOS: 1 - 2

Figure 4 provides PS-1 Oligonucleotide Probes. SEQ ID NOS: 3 - 5

Figure 5 provides tabulated results of quantification of the ISH signal for PS-1-long and PS-1-short mRNAs in human brain.

DETAILED DESCRIPTION OF THE INVENTION

The presenilin 1 (herein "PS-1") gene encodes a neuropeptide predicted to be a classical seven transmembrane protein (Sherrington et al. Nature 1995, 375:754-760). Missense mutations within this gene have been found in several families exhibiting early-onset Alzheimer's disease. Genomic analysis has revealed the intron-exon boundaries of the hnRNA. A common polymorphism located within the intron 3' to exon 9 was identified in early onset AD patients. This polymorphism also showed a strong association with the occurrence of typical late onset AD families. This particular mutation did not produce an alteration in the coding sequence but is typical of variations leading to alternatively spliced proteins.

Other mutations within different introns of the PS-1 gene have been identified. These lead to alternatively spliced variants as well. One novel variant of the PS-1 protein isolated from a human cerebellar cDNA library contains a four amino acid insertion between codons 26 and 27 (VRSQ) (Figure 3). This variant arises from alternative use of a 5' exon donor site in the exon 3/intron 3 boundary and results in the loss of some potential phosphorylation sites. A similar motif (VRXQ- where X is a hydrophilic amino acid) arising from aberrant splicing has also arisen due to alternative splicing in several other neurological proteins as well.

For example, the mRNA for tyrosine hydroxylase, the rate limiting enzyme in the synthesis of catecholamines, can undergo alternative splicing to produce several different isoforms (Kobayashi et al. J. Biochem. 1988, 103(6) 907-12; Lewis et al. Neuroscience 1993, 54(2) 477-92). The identified variants contain a 12 bp insertion encoding the sequence VRGQ. Isoforms containing the VRGQ insertion have also been found to exhibit alterations in phosphorylation by MAP kinase (Sutherland et al. Eur J Biochem. 1993, 217(2) 715-22). Furthermore, a tyrosine hydroxylase variant containing this insertion has been implicated in Parkinson's disease.

Another neuropeptide, gamma-Aminobutyric acid A (GABAA) receptor, undergoes alternative splicing to yield a

multiplicity of transcripts (Whiting et al. P.N.A.S. 1990, 87(24) 9966-70; Lasham et al. Biochem. Soc. Trans. 1991, 19 (1) 9S). GABA receptors are multisubunit ligand gated ion channels which mediate neuronal inhibition by GABAA and are composed of at least four subunit types (alpha, beta, gamma, and delta). The beta 4 subunit can undergo alternative splicing at two 5'-donor splice sites separated by 12 bp in the region that encodes the presumed intracellular loop between transmembrane domains M3 and M4. The insertion of the 12 bp sequence results in the addition of a VREQ motif (Bateson et al. J. Neurochem 1991, 56(4) 1437-40).

In all three neurological proteins, the alternative splice site generates variants containing a specific motif (VRXQ) which appears to be intracellularly located and alters phosphorylation by various kinases.

In the present invention, a method for detecting the presence of the VRXQ motif in polyadenylated messenger RNA transcripts (polyA mRNA) and resultant expressed proteins, (where V is valine, R is arginine, X is any hydrophilic amino acid residue, and Q is glutamine) or in cDNA resulting from these RNAs is provided. A method for quantitating such transcripts encoding and proteins having a VRXQ motif are also provided. Oligonucleotides having the anticodon sequences associated with the VRXQ motif having degenerate positions at the third base position of each codon can be used for the detection and quantitation of mRNA. Additionally, these oligonucleotides can be associated with codon sequences and used for the detection of cDNAs, and quantitation of the transcript from which the cDNA was derived. For example, codon and anticodon oligonucleotides for VRNQ comprise GU(N) AG(A/G) AA(C/U) CA(A/G) and the reverse complement. Hybridization of appropriate oligonucleotides can be detected and quantitated directly by procedures well known to those of skill in the art using radioactively or fluorescently labeled oligonucleotides. Indirect detection and quantitation procedures such as, but not limited to, biotinylated oligonucleotides/streptavidin-horseradish peroxidase, enhanced chemiluminescent detection, or fluorescently tagged streptavidins can also be performed.

Specific antibodies against the VRXQ motif can also be used for detection of the motif and quantitation of proteins having the motif. Various procedures known in the art may be used for the production of such antibodies.

For example, these antibodies can be obtained by direct injection of a polypeptide containing a VRXQ motif into an animal, preferably a nonhuman. The antibody so obtained will then bind to polypeptides containing this motif. Such antibodies can then be used to isolate and quantitate polypeptides containing this motif from tissues.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, Nature 1975, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology Today 1983, 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., 1985, pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to the immunogenic motif of this invention. Also, transgenic mice may be used to express humanized antibodies to polypeptides containing this motif.

Primary antibody-antigen reactions can be visualized and quantitated secondarily by standard enzyme-linked immunosorbent assay (ELISA) procedures. An ELISA assay initially comprises preparing an antibody specific to a VRXQ motif, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as horse radish peroxidase. A sample is then removed from a host and incubated on a solid support, e.g., a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein like BSA. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attach to any proteins containing the VRXQ motif attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is then placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to proteins containing the VRXQ motif. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of protein containing the VRXQ motif present in a given volume of patient sample when compared against a standard curve to detect and quantitate the protein. Examples of other detectable reagents which can be used include, but are not limited to, luciferase and fluorescently or radioactively tagged secondary antibodies. Specific populations of immune cells or chimeric cells (e.g., hybridomas) that express antibodies to VRXQ epitopes on their cell surfaces and respond by degranulation or release of cellular contents such as histamines that can be detected functionally or preloaded radiolabeled metals such as chromium are also useful.

Embodiments of the invention can be used to detect alterations in and make comparisons between expression in of PS-1 variants in presumptive neurodegenerative disease, particularly neurodegenerative disease associated with head injury and AD, and more particularly chromosome 14 FAD. In a particularly preferred embodiment, probes and methods of the invention can be used to detect a reduction in the expression of PS-1 transcript encoding the VRSQ motif, shown by this invention to be a diagnostic marker for chromosome 14 FAD, since lowered levels are associated with chromosome 14 FAD. Preferred embodiments of the invention provide for comparisons between variants comprising the VRSQ region with those lacking it enabling the diagnosis of AD, particularly chromosome 14 FAD.

The methods of the invention to detect and quantitate PS-1 polynucleotide sequence, PS-1 expression levels and

gene expression products, particularly the immunological methods and methods using oligonucleotides, can be used with bodily tissues and fluids from individuals. Preferred bodily tissues and fluids useful with the methods of the invention include, but are not limited to, blood cells, plasma, skin cells, and brain cells, particularly neuronal, glial, and astrocyte cells.

5 The following examples are provided for illustrative purposes only and are not intended to limit the invention.

EXAMPLES

Example 1

10

A novel splice variant of the PS-1 gene described by Sherrington et al. Nature 1995, 375:754-760, was isolated from a human cerebellar and a human fibroblast library. In this novel splice variant there is a deletion of four amino acids at codons 26-27 (VRSQ). This arises from alternative use of a 5' exon donor site in the exon3/intron 3 (-52 to 75 nt) boundary. The ...CAG/gta... boundary of the final Gln codon of exon 3 of the VRSQ motif provides a 5' exon AG donor site and GT intron consensus 5' boundary and use of this splice site results in the insertion of the 12-nts encoding the VRSQ motif. The upstream ...ACT/GTA... boundary of the Thr-Val codons provides the less preferred CT (AG preferred) 5' exonic boundary to the consensus GT 5' intronic boundary and splicing at this site would remove the VRSQ motif. Interestingly, in the PS-1 protein of Sherrington et al. Nature 1995, 375:754-760, this is the sole observed product and point mutations are interspersed elsewhere.

20

Example 2

In the GABA receptor 4 subunit alternative splicing adds a VREQ motif (Bateson et al. J. Neurochem 1991, 56(4) 1437-40). A chicken genomic cDNA library was screened with chicken beta-4' subunit cDNA at high stringency. Southern blot analysis, using cDNA sequence specific oligonucleotides as probes and subsequent restriction mapping allowed the identification of overlapping DNA fragments containing the coding regions of the beta-4 subunit gene. These fragments were subcloned into pBluescript and sequenced. Complete sequencing of one of the clones revealed the presence of 12 bp in the part encoding the intracellular loop (amino acid residues 335-338). Analysis of the beta-4 subunit gene reveals that the different transcripts encoding the two variants (absence or presence of 12bp loop) arise by the use of one of two 5'-donor splice sites (located in the intron immediately 3' of the 12 bp sequence).

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Example 3

The expression of two PS-1 mRNA transcripts, one containing (herein "PS-1-long") and one lacking the VSRQ motif (herein "PS-1-short"), in the brains of patients with early onset FAD was analyzed. In situ hybridization (ISH) was used to determine the qualitative and quantitative pattern of expression of PS-1 mRNA in the brains of early onset (presumptive chromosome 14-linked) FAD cases; comparisons with brains from patients with late onset AD and from normal individuals were made.

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40 In Situ Hybridization

PS-1 mRNA expression was examined in 4 neurologically normal control cases, 6 late onset AD cases and 3 early onset FAD cases. The late onset cases were thought to be of a sporadic nature as there was no evidence of family history and the mean age at death was 81.2 years (range: 79-84 years); they had a mean post mortem delay of 8.3 hours. The early onset FAD cases were presumed to be linked to chromosome 14 as they all had onset ages, family history, clinical presentations and histopathology typical of chromosome 14-linked FAD. For these the mean age at death was 45 years (range: 44-46 years) and the mean post mortem delay was 41.7 hours. All AD cases were diagnosed according to standard pathological criteria (Khachaturian, 1985, Archives of Neurology, 42:1097-1105). The controls had a mean age at death of 68.8 years (range: 57-85 years) and mean post mortem delay of 11.8 hours. The brain regions examined were the hippocampus, temporal cortex and frontal cortex (regions severely affected by AD pathology), the visual cortex (an area relatively unaffected, but which at the time of death may be in the early stages of the disease process) and the cerebellum (an area not affected by the classic pathology associated with AD and with no clinical involvement).

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Three different oligoprobes were chosen and synthesized (Figure 4): one to detect PS-1-long, one to PS-1-short and one that recognizes both transcripts, PS-1-both. These probes are not predicted to detect the transcripts of presenilin-2, a closely related gene on chromosome 1 (Rogaev, et al., 1995, Nature 376:775-78).

55

The ISH methodology is well known in the art and has been described in detail elsewhere (Najlerahim et al., 1990, FEBS Letters 7:317-333). For the ISH analyses 10(m cryostat tissue sections were used. Probes were labelled at their

3' end with ³⁵S-dATP using the NEN DuPont 3' end labelling system. Hybridization and wash temperatures for the various probes are given in Figure 4. Hybridized sections were apposed to tritium-sensitive film for the generation of autoradiographs. Hybridization with the PS-1 probes in the sense orientation on adjacent sections were used to control for non-specific background. The signal on autoradiographic film was quantified using an image analyzer (Seescan®).
 5 A representative area over most of a tissue section was measured: for example, in the hippocampus the different subfields were not separately quantified. The background signal (sense strand hybridization) was subtracted from the antisense signal. Statistical analysis of the data was performed using the well known two-tailed Student's t-test.

Northern Analysis

10 Northern analysis was carried out with the PS-1-both probe on a Northern blot (Clontech®, catalogue number: 7750-1) containing polyA+ mRNA from a number of different human brain regions. The probe was 3' end labelled with ³²P-dATP using terminal transferase and hybridized under standard conditions (Clontech®, data sheet).

15 Diagnostic Methods and Reagents for FAD

In situ hybridization using all three probes revealed that PS-1 mRNA was present in all of the brain regions examined. Hybridization with a sense strand control probe gave a very low background signal. In the cerebral cortex (three regions) a signal was detected in both the grey and white matter, often with a similar intensity. A diffuse rather than
 20 laminar pattern was observed in grey matter and in the hippocampus the different subfields were not readily delineated (although the dentate gyrus was sometimes visible). In the cerebellum, the granule cell layer contained the most labelling. These data are consistent with PS-1 mRNA expression in both neurons and glia.

Northern analysis confirmed that the PS-1-both oligoprobe detected a major transcript in human brain of the correct size for PS-1 mRNA (in accordance with the sequence data of Sherrington et al, 1995, Nature 375:754-760). A major
 25 band of approximately 3.4 kb was detected in all brain regions examined, indicating a wide distribution in brain for PS-1 mRNA. The observation of PS-1 mRNA in corpus callosum is consistent with the interpretation from our ISH data that PS-1 is expressed in glia.

A similar anatomical pattern was seen by ISH, in each region, for both PS-1-long and PS-1-short transcripts. Nevertheless there appeared to be differences between the transcripts in their levels of expression according to brain
 30 region; for example PS-1-short was relatively less abundant in the cerebellum (Figure 5).

The hybridization pattern was similar for the controls, sporadic AD and FAD cases. Quantification of the autoradiographic film revealed a statistically significant reduction in the amount of PS-1-long mRNA in FAD hippocampus and frontal cortex compared with the sporadic AD cases (Figure 5; $p = 0.003$ and $p = 0.014$ respectively). In the cerebellum there was no significant difference between the controls, sporadic AD and FAD cases. The reduction in PS-1-long
 35 appears to be specific because there was no change in the level of expression of PS-1-short mRNA in any brain region investigated between the three different groups (Figure 5), which indicates reasonable data consistency.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: University of South Florida, Washington University and SmithKline Beecham Corporation

(ii) TITLE OF INVENTION: A Novel Diagnostic Marker for Splicing Variants of Genes Associated with Neurological Function

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb

STORAGE

(B) COMPUTER: IBM 486

(C) OPERATING SYSTEM: WINDOWS FOR WORKGROUPS

(D) SOFTWARE: WORDPERFECT 5.1

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1914

EP 0 791 660 A1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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 10 TCCAGAATGC ACAGATGTCT GAGGACAACC ACCTGAGCAA TACTAATGAC 300
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 GCCATTATCT AATGGACGAC CCCAGGGTAA CTCCCGGCAG GTGGTGGAGC 400
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 15 GTGATCATGC TCTTGTCCC TGTGACTCTC TGCATGGTGG TGGTCGTGGC 500
 TACCATTAAG TCAGTCAGCT TTTATACCCG GAAGGATGGG CAGCTAATCT 550
 ATACCCCATC CACAGAAGAT ACCGAGACTG TGGGCCAGAG AGCCCTGCAC 600
 TCAATTCTGA ATGTGCCAT CATGATCAGT GTCATTGTTG TCATGACTAT 650
 20 CCTCCTGGTG GTTCTGTATA AATACAGGTG CTATAAGGTC ATCCATGCCT 700
 GGCTTATTAT ATCATCTCTA TTGTTGCTGT TCTTTTTC ATTCAATTAC 750
 TTGGGGGAAG TGTTTAAAC CTATAACGTT GCTGTGGACT ACATTACTGT 800
 TGCACCTCTG ATCTGGAATT TTGGTGTGGT GGAATGATT TCCATTCACT 850
 25 GGAAAGGTCC ACTTCGACTC CAGCAGGCAT ATCTCATTAT GATTAGTGCC 900
 CTCATGGCCC TGGTGTATAT CAAGTACCTC CCTGAATGGA CTGCGTGGCT 950
 CATCTTGGCT GTGATTTCAG TATATGATTT AGTGGCTGTT TTGTGTCCGA 1000
 AAGGTCCACT TCGTATGCTG GTTGAAACAG CTCAGGAGAG AGATGAAACG 1050
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 50 ACGRTTTTGA MCATACTTCA TCGCAGTGGA CTGTGTCCCT CGGTGCAGAA 1850
 ACTACCAGAT TTGAGGGACG AGGTCAAGGA GATATGATAG GCCCGGAAGT 1900
 TGCTGTGCCC ATCA

1914

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 463

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

MET THR GLU LEU PRO ALA PRO LEU SER TYR PHE GLN ASN ALA GLN			
1	5	10	15
MET SER GLU ASP ASN HIS LEU SER ASN THR ASN ASP ASN ARG GLU			
	20	25	30
ARG GLN GLU HIS ASN ASP ARG ARG SER LEU GLY HIS PRO GLU PRO			
	35	40	45
LEU SER ASN GLY ARG PRO GLN GLY ASN SER ARG GLN VAL VAL GLU			
	50	55	60
GLN ASP GLU GLU GLU ASP GLU GLU LEU THR LEU LYS TYR GLY ALA			
	65	70	75
LYS HIS VAL ILE MET LEU PHE VAL PRO VAL THR LEU CYS MET VAL			
	80	85	90
VAL VAL VAL ALA THR ILE LYS SER VAL SER PHE TYR THR ARG LYS			
	95	100	105
ASP GLY GLN LEU ILE TYR THR PRO PHE THR GLU ASP THR GLU THR			
	110	115	120
VAL GLY GLN ARG ALA LEU HIS SER ILE LEU ASN ALA ALA ILE MET			
	125	130	135
ILE SER VAL ILE VAL VAL MET THR ILE LEU LEU VAL VAL LEU TYR			
	140	145	150
LYS TYR ARG CYS TYR LYS VAL ILE HIS ALA TRP LEU ILE ILE SER			
	155	160	165
SER LEU LEU LEU LEU PHE GLU GLU SER PHE ILE TYR LEU GLY GLU			
	170	175	180

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	VAL PHE LYS THR TYR ASN VAL ALA VAL ASP TYR ILE THR VAL ALA		
	185	190	195
5	LEU LEU ILE TRP ASN PHE GLY VAL VAL GLY MET ILE SER ILE HIS		
	200	205	210
10	TRP LYS GLY PRO LEU ARG LEU GLN GLN ALA TYR LEU ILE MET ILE		
	215	220	225
15	SER ALA LEU MET ALA LEU VAL PHE ILE LYS TYR LEU PRO GLU TRP		
	230	235	240
20	THR ALA TRP LEU ILE LEU ALA VAL ILE SER VAL TYR ASP LEU VAL		
	245	250	255
25	ALA VAL LEU CYS PRO LYS GLY PRO LEU ARG MET LEU VAL GLU THR		
	260	265	270
30	ALA GLN GLU ARG ASP GLU THR LEU PHE PRO ALA LEU ILE TYR SER		
	275	280	285
35	SER THR MET VAL TRP LEU VAL ASN MET ALA GLU GLY ASP PRO GLU		
	290	295	300
40	ALA GLN ARG ARG VAL SER LYS ASN SER LYS TYR ASN ALA GLU SER		
	305	310	315
45	THR GLU ARG GLU SER GLN ASP THR VAL ALA GLU ASN ASP ASP GLY		
	320	325	330
50	GLY PHE SER GLU GLU TRP GLU ALA GLN ARG ASP SER HIS LEU GLY		
	335	340	345
55	PRO HIS ARG SER THR PRO GLU SER ARG ALA ALA VAL GLN GLU LEU		
	350	355	360
	SER SER SER ILE LEU ALA GLY GLU ASP PRO GLU GLU ARG GLY VAL		
	365	370	375
	LYS LEU GLY LEU GLY ASP PHE ILE PHE TYR SER VAL LEU VAL GLY		
	380	385	390
	LYS ALA SER ALA THR ALA SER GLY ASP TRP ASN THR THR ILE ALA		
	395	400	405

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5 CYS PHE VAL ALA ILE LEU ILE GLY LEU CYS LEU THR LEU LEU LEU
410 415 420

LEU ALA ILE PHE LYS LYS ALA LEU PRO ALA LEU PRO ILE SER ILE
425 430 435

10 THR PHE GLY LEU VAL PHE TYR PHE ALA THR ASP TYR LEU VAL GLN
440 445 450

15 PRO PHE MET ASP GLN LEU ALA PHE HIS GLN PHE TYR ILE
455 460

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCACCTCAATT CTGAATGCTG CCATCATGAT

30

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGCAATACTG TACGTAGCCA GAATGACAAT

30

(2) INFORMATION FOR SEQ ID NO: 5:

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(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CACCTGAGCA ATACWATGAC AATAGAGAA

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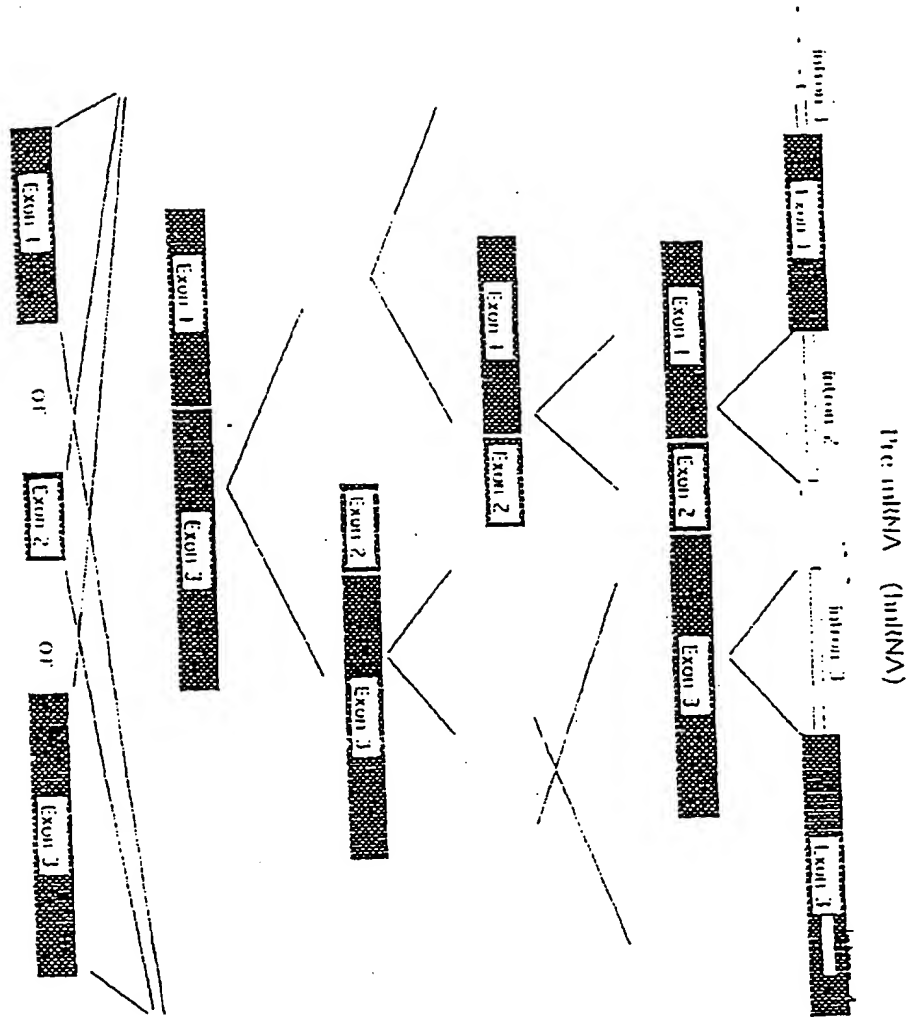
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Possible Final mRNAs

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Figure 1



Structure of Exon-Intron Boundaries and Intron Consensus Sequences

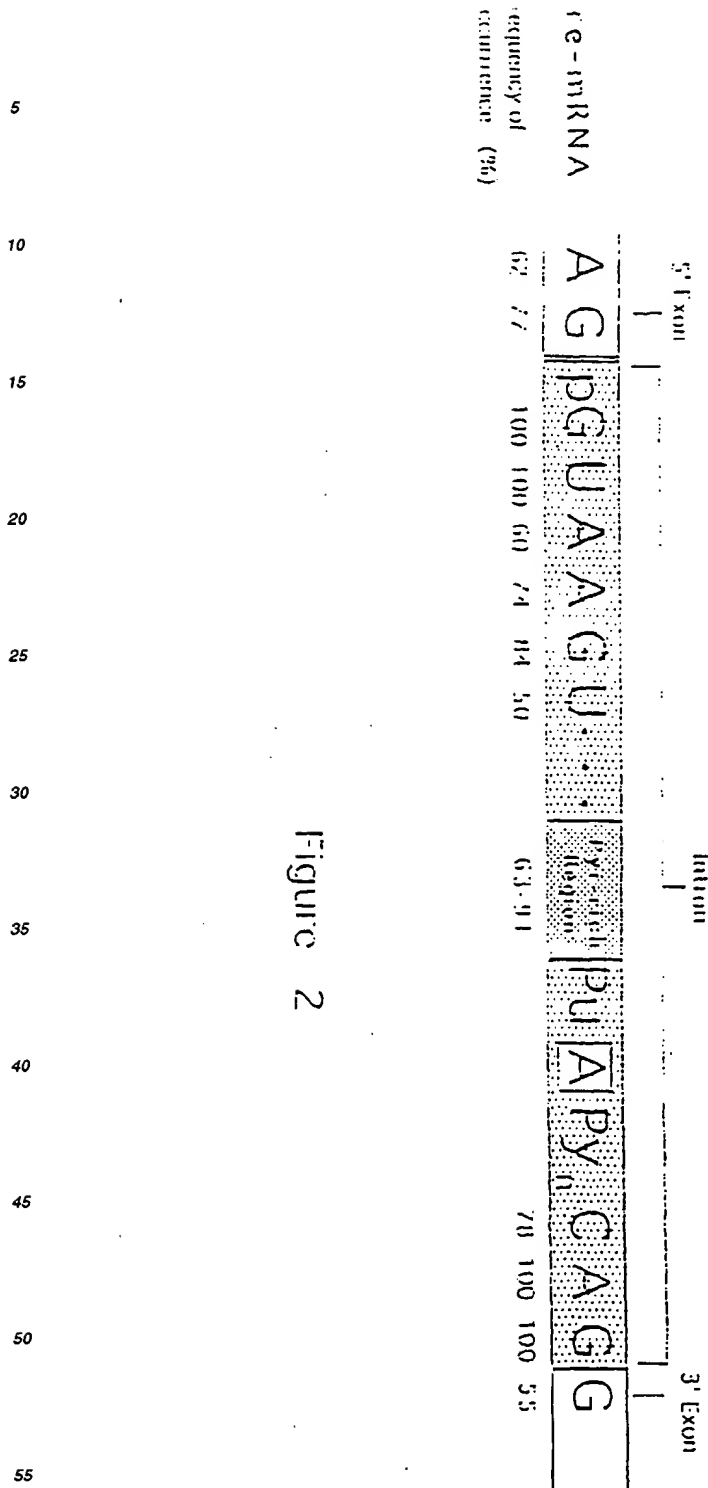


Figure 2

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FIGURE 1A

5

11 20 29 38 47 56
5' CCG TAC GTA GCC GCG GCG GCA GCG GGG CGG CGG GGA AGC GTA TGC ATA CAA ATT

10

65 74 83 92 101 110
TAT TAG CAT GCA GAC TGG GAG AAC CAC AAG ACC TAA TCT GGG AGC CTG CAA GTG

15

119 128 137 146 155 164
ACA ACA GCC TTT GCG GTC CTT AGA CAG CTT GGC CTG GAG GAG AAC ACA TGA AAG

20

173 182 191 200 209 210
AAA GAA CCT CAA GAG GCT TTG TTT TCT GTG AAA CAG TAT TTC TAT ACA GTT GCT

25

227 236 245 254 263 272
CCA ATG ACA GAG TTA CCT GCA CCG TTG TCC TAC TTC CAG AAT GCA CAG ATG TCT

30

M T E L P A P L S Y F Q N A Q M S

35

281 290 299 308 317 326
GAG GAC AAC CAC CTG AGC AAT ACT AAT GAC AAT AGA GAA CGG CAG GAG CAC AAC

E D N H L S N T N D N R E R Q E H N

40

335 344 353 362 371 380
GAC AGA CGG AGC CTT GGC CAC CCT GAG CCA TTA TCT AAT GGA CGA CCC CAG GGT

D R R S L G H P E P L S N G R P Q G

45

389 398 407 416 425 434
AAC TCC CGG CAG GTG GTG GAG CAA GAT GAG GAA GAA GAT GAG GAG CTG ACA TTC

N S R Q V V E Q D E E E D E E L T L

50

443 452 461 470 479 488
AAA TAT GGC GCC AAG CAT GTG ATC ATG CTC TTT GTC CCT GTG ACT CTC TGC ATG

K Y G A K H V I H L F V P V T L C M

55

497 506 515 524 533 542
GTG GTG GTC GTG GCT ACC ATT AAG TCA GTC AGC TTT TAT ACC CGG AAG GAT GGG

V V V V A T I K S V S F Y T R K D G

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FIGURE 1B

		551				560			569			578			587			596
5	CAG	CTA	ATC	TAT	ACC	CCA	TTC	ACA	GAA	GAT	ACC	GAG	ACT	GTG	GGC	CAG	AGA	GCC
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	Q	L	I	Y	T	P	F	T	E	D	T	E	T	V	G	Q	R	A
			605			614			623			632			641			650
10	CTG	CAC	TCA	ATT	CTG	AAT	GCT	GCC	ATC	ATG	ATC	AGT	GTC	ATT	GTT	GTC	ATG	ACT
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	L	H	S	I	L	N	A	A	I	M	I	S	V	I	V	V	M	T
			659			668			677			686			695			704
15	ATC	CTC	CTG	GTG	GTT	CTG	TAT	AAA	TAC	AGG	TGC	TAT	AAG	GTC	ATC	CAT	GCC	TGG
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	I	L	L	V	V	L	Y	K	Y	R	C	Y	K	V	I	H	A	W
			713			722			731			740			749			758
20	CTT	ATT	ATA	TCA	TCT	CTA	TTG	TTG	CTG	TTC	TTT	TTT	TCA	TTC	ATT	TAC	TTG	GGG
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	L	I	I	S	S	L	L	L	L	F	E	F	S	F	I	Y	L	G
			767			776			785			794			803			812
25	GAA	GTG	TTT	AAA	ACC	TAT	AAC	GTT	GCT	GTG	GAC	TAC	ATT	ACT	GTT	GCA	CTC	CTG
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	E	V	F	K	T	Y	N	V	A	V	D	Y	I	T	V	A	L	L
			821			830			839			848			857			866
30	ATC	TGG	AAT	TTT	GGT	GTG	GTG	GGA	ATG	ATT	TCC	ATT	CAC	TGG	AAA	GGT	CCA	CTT
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	J	W	N	F	G	V	V	G	M	I	S	I	H	W	K	G	P	L
			875			884			893			902			911			920
35	CGA	CTC	CAG	CAG	GCA	TAT	CTC	ATT	ATG	ATT	AGT	GCC	CTC	ATG	GCC	CTG	GTG	TTT
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	R	L	Q	Q	A	Y	L	I	M	I	S	A	L	M	A	L	V	F
			929			938			947			956			965			974
40	ATC	AAG	TAC	CTC	CCT	GAA	TGG	ACT	GCG	TGG	CTC	ATC	TTG	GCT	GTG	ATT	TCA	GTA
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	I	K	Y	L	P	E	W	T	A	W	L	I	L	A	V	I	S	V
			983			992			1001			1010			1019			1028
45	TAT	GAT	TTA	GTG	GCT	GTT	TTG	TGT	CCG	AAA	GGT	CCA	CTT	CGT	ATG	CTG	GTT	GAA
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	Y	D	L	V	A	V	L	C	P	K	G	P	L	R	M	L	V	E
			1037			1046			1055			1064			1073			1082
50	ACA	GCT	CAG	GAG	AGA	GAT	GAA	ACG	CTT	TTT	CCA	GCT	CTC	ATT	TAC	TCC	TCA	ACA
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	T	A	Q	E	R	D	E	T	L	F	P	A	L	I	Y	S	S	T
			1091			1100			1109			1118			1127			1136
55	ATG	GTG	TGG	TTG	GTG	AAT	ATG	GCA	GAA	GGA	GAC	CCG	GAA	GCT	CAA	AGG	AGA	GTA

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FIGURE 1C

5	M	V	W	L	V	N	M	A	E	G	D	P	S	A	Q	R	R	V
		1145			1154			1163			1172			1181			1190	
	TCC	AAA	AAT	TCC	AAG	TAT	AAT	GCA	GAA	AGC	ACA	GAA	AGG	GAG	TCA	CAA	GAC	ACT
10	S	K	N	S	K	Y	N	A	E	S	T	E	R	E	S	Q	D	T
		1199			1208			1217			1226			1235			1244	
	GTT	GCA	GAG	AAT	GAT	GAT	GGC	GGG	TTC	ACT	GAG	GAA	TGG	GAA	CCC	CAG	AGG	GAC
15	V	A	E	H	D	D	G	G	F	S	E	E	W	E	A	Q	R	D
		1253			1262			1271			1280			1289			1298	
	AGT	CAT	CTA	GGG	CCT	CAT	CGC	TCT	ACA	CCT	GAG	TCA	CGA	GCT	GCT	CTC	CAG	GAA
20	S	H	L	G	P	H	R	S	T	P	E	S	R	A	A	V	Q	E
		1307			1316			1325			1334			1343			1352	
	CTT	TCC	AGC	AGT	ATC	CTC	GCT	GGT	GAA	GAC	CCA	GAG	GAA	AGG	GGA	GTA	AAA	CTT
25	L	S	S	S	I	L	A	G	E	D	P	E	E	R	G	V	K	L
		1361			1370			1379			1388			1397			1406	
	GGA	TTG	GGA	CAT	TTC	ATT	TTC	TAC	AGT	GTT	CTG	GTT	GGT	AAA	GCC	TCA	GCA	ACA
30	G	L	G	D	F	I	F	Y	S	V	L	V	G	K	A	S	A	T
		1415			1424			1433			1442			1451			1460	
	GCC	AGT	GGA	GAC	TGG	AAC	ACA	ACC	ATA	GCC	TGT	TTC	GTA	GCC	ATA	TTA	ATT	GGT
35	A	S	G	D	W	N	T	T	I	A	C	F	V	A	I	L	I	G
		1469			1478			1487			1496			1505			1514	
	TTG	TGC	CTT	ACA	TTA	TTA	CTC	CTT	GCC	ATT	TTC	AAG	AAA	GCA	TTG	CCA	GCT	CTT
40	I	C	L	T	L	L	L	L	A	I	F	K	K	A	L	P	A	L
		1523			1532			1541			1550			1559			1568	
	CCA	ATC	TCC	ATC	ACC	TTT	GGG	CTT	GTT	TTC	TAC	TTT	GCC	ACA	GAT	TAT	CTT	GTA
45	P	I	S	I	T	F	G	L	V	F	Y	F	A	T	D	Y	L	V
		1577			1586			1595			1604			1613			1622	
	CAG	CCT	TTT	ATG	GAC	CAA	TTA	GCA	TTC	CAT	CAA	TTT	TAT	ATC	TAG	CAT	ATT	TGC
50	Q	P	F	M	D	Q	L	A	F	H	Q	F	Y	I	*			
		1631			1640			1649			1658			1667			1676	
	GGT	TAG	AAT	CCC	ATG	GAT	GTT	TCT	TCT	TTG	ACT	ATA	ACA	AAA	TCT	GGG	GAG	GAC
55																		

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FIGURE 1D

5
1685 1694 1703 1712 1721 1730
AAA GGT GRT TTT CCT GTG TCC CAC ATC TAA CAA AGT CAA GAT TCC CGR CTG GAC

10
1739 1748 1757 1766 1775 1784
TTT TGC AGC TTC CTK CCA AGT CTT CCT GAC CAC CTT GCA CTH TTG GAC TTT GGA

15
1793 1802 1811 1820 1829 1838
RGG AGG TGC CTA KAG AAA ACG RTT TTG AMC ATA CTT CAT CGC AGT GGA CTG TGT

20
1847 1856 1865 1874 1883 1892
CCC TCG GTG CAG AAA CTA CCA GAT TTG AGG GAC GAG GTC AAG GAG ATA TGA TAG

25
1901 1910
GUC CGG AAG TTG CTG TGC CCA TCA 3'

Figure 4

PS-1 Oligonucleotide Probes

Probe	Sense Sequence	Bases *	Ti °C	Tw °C
PS-1-both	5'-GCACTCAATTCTGAATGCTGCCATCATGAT-3'	638-667	24	50
	SEQ ID NO: 3			
PS-1-long	5'-AGCAATACTGTACGTAGCCAGAATGACAAT-3'	315-344	23	49
	SEQ ID NO: 4			
PS-1-short	5'-CACCTGAGCAATACT/AATGACAATAGAGAA-3'	309-323 and 336-350	22	47
	SEQ ID NO: 5			

*Refers to EMBL and GenBank entry HUMS182R (accession number: L42110); Sherrington et al 1995, Nature 375:754-760. Ti represents the hybridization temperature (incubation) and Tw represents the wash temperature. The underlined bases code for the amino acids V, R, S and Q.

Figure 5.**Quantification of the ISH signal for PS-1-long and PS-1-short mRNAs in human brain.**

Brain region	Case	PS-1-long (n)	PS-1-short (n)
Hippocampus	Control	0.025 ± 0.014 (2)	0.023 (1)
	AD	0.035 ± 0.007 (3)	0.026 ± 0.01 (3)
	FAD	0.008 ± 0.001 (3)*	0.030 ± 0.004 (3)
Frontal cortex	AD	0.024 ± 0.005 (3)	0.042 ± 0.014 (3)
	FAD	0.012 ± 0.0 (3)**	0.022 ± 0.011 (3)
Cerebellum	Control	0.036 (1)	0.013 (1)
	AD	0.024 ± 0.007 (3)	0.019 ± 0.006 (3)
	FAD	0.012 ± 0.002 (2)	0.014 ± 0.005 (2)
Temporal cortex	FAD	0.014 ± 0.009 (3)	0.015 ± 0.01 (3)
Visual Cortex	FAD	0.016 ± 0.007 (3)	0.032 ± 0.001 (3)

values represent means ± s.d.; units are arbitrary (machine grey levels). *FAD vs AD p = 0.003; **FAD vs AD p = 0.014; Student's t-test.

Claims

1. A method of identifying an individual susceptible to a neurological disease comprising:

providing a sample of genetic material from an individual susceptible to a neurological disease; and detecting the presence of an alternative splice site comprising the sequence VRXQ, wherein V is valine, R is arginine, X is any hydrophobic amino acid residue and Q is glutamine, in a polyadenylated messenger RNA transcript or protein encoded therefrom in the sample of genetic material.

2. The method of claim 1 wherein the sequence VRXQ is detected using selected oligonucleotide probes comprising anticodon sequences associated with the sequence VRXQ having degenerate positions at the third base position.
3. The method of claim 2 further comprising associating said oligonucleotides with codon sequences and detecting cDNA.

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4. The method of claim 1 wherein the sequence VRXQ is detected using an antibody against a polypeptide comprising the sequence VRXQ.
- 5 5. The method of claim 1 wherein the neurological disease comprises Alzheimer's Disease and the mRNA or protein is encoded by the presenilin 1 gene.
6. The method of claim 5 wherein the sequence comprises a 4 amino acid insertion between codons 26 and 27 of the gene and the sequence VRSQ.
- 10 7. The method of claim 1 wherein the mRNA or protein is encoded by the gamma-Aminobutyric acid A receptor gene and the sequence comprises VREQ.
8. The method of claim 1 wherein the mRNA or protein is encoded by the tyrosine hydroxylase gene and the sequence comprises VRGQ.
- 15 9. A method for diagnosing a neurological disease comprising determining the levels of polyadenylated messenger RNA transcripts or proteins encoded therefrom comprising the sequence VRXQ wherein V is valine, R is arginine, X is any hydrophobic amino acid residue and Q is glutamine, in a sample of genetic material and comparing these levels with established controls.
- 20 10. The method of claim 9 wherein the neurological disease comprises Alzheimer's Disease and the mRNA or protein is encoded by the presenilin 1 gene.
11. The method of claim 10 wherein the sequence comprises a 4 amino acid insertion between codons 26 and 27 of the gene and the sequence VRSQ.
- 25 12. The method of claim 9 wherein the mRNA or protein is encoded by the gamma-Aminobutyric acid A receptor gene and the sequence comprises VREQ.
13. The method of claim 9 wherein the mRNA or protein is encoded by the tyrosine hydroxylase gene and the sequence comprises VRGQ.
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European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 97300988.9

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 6)
A, D	NATURE GENETICS, vol. 8, no. 1, October 1994 S.G. KALER et al. "Occipital horn syndrome and a mild Menkes phenotype associated with splice site mutations at the MNK locus" pages 195-202 * Totality *	1, 9	C 12 Q 1/68
A, D	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 91, September 1994 J.P. DISANTO et al. "Defective human interleukin 2 receptor gamma chain in an typical X chromosome-linked severe combined immunodeficiency with peripheral T cells" pages 9466-9470 * Totality *	1, 9	
			TECHNICAL FIELDS SEARCHED (Int. Cl. 6)
A, D	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 202, no. 1, 1994 T. HAMAGUCHI et al. "A new variant of muscle phosphofructokinase deficiency in a Japanese case with abnormal RNA splicing" pages 444-449 * Totality *	1, 9	C 12 Q
A	WO - A - 94/18 319 (INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE) * Claims 1-6, 12-15 *	1, 9	
The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 02-06-1997	Examiner WOLF
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

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